REAL-TIME PCR ASSAY FOR DETECTION OF TETRACYCLINE RESISTANCE GENES OF GRAM-NEGATIVE BACTERIA

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ABSTRACT

Doxycycline, a new generation tetracycline antibiotic, is currently one of choice for the treatment and prevention of infections caused by agents of biowarfare. We are developing real time PCR assays to detect tetracycline resistance genes in Gram-negative bacteria. The assay was developed as a multiplex SYBR Green I detection using the Roche Lightcycler and multi-melting peak analysis followed by a specific 5' nuclease assay. Specific primer pairs were selected for the PCR amplification of seven tetracycline resistance genes commonly found in Gram-negative organisms. A combination of primer pairs were used in a multiplex PCR reaction with SYBR Green I to detect a group of Tet resistance genes: *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G) and *tet*(H). Based on the melting peak analysis of our SYBR Green I reaction, we could preliminarily determine the class of the Tet resistance determinant that gave the positive signal. To confirm the result, we designed specific TaqMan primers and probes for each class of Tet determinant. Positive results were determined by comparing the Ct (crossing point) as well as DNA sequencing. We analyzed forty-eight clinical isolates by both assays. In 37 samples, the 5' nuclease assay confirmed the identity shown in SYBR Green I multiplex PCR reaction. 4 samples confirmed to be one uncommon *tet* gene (Tet J resistance determinant). Other 6 keep unknown. Our results demonstrate that the multiplex PCR assay with SYBR Green I is a method of significant saving in terms of labor and time in strains analysis. The SYBR Green I assay coupled with a class-specific 5' nuclease assay is a two-fold confirmation and identification of the Tet resistance genes present in Gram-negative organisms.

INTRODUCTION

Doxycycline, a new generation tetracycline antibiotic, is currently one of choice for prophylactic treatment of bacterial disease. Tetracycline resistance determinants are wide spread among bacterial species and have been identified in as many as 32 Gram negative and 22 Gram-positive organisms. Further more in the multi-drug resistant bacteria. Bacterial resistance to tetracycline is mediated mainly by two mechanisms; the use of an energy-dependent efflux pump

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of tetracycline, or altering the ribosome to prevent effective binding of the tetracycline. A third mechanism, enzymatic inactivation of tetracycline, is relatively uncommon. Historically, the genotyping of tetracycline resistance genes in bacterial isolations has been performed by hybridization with probes generated from known tetracycline resistance determinants [(1)]. This method is not sufficiently sensitive for environmental studies [(2)], but has lead to the identification of Tet resistance gene classes based on over 80 % DNA homology. [(3)] More recently, a PCR-based approach has been developed for detection of tetracycline efflux pumps of Gram-negative bacteria in environmental samples and in bacterial isolates [(4)]. Real-time, quantitative PCR has reported comparable sensitivity but superior reproducibility and precision compared to previous methods [(5)]. The fluorescence-based real-time assays allow for multiple PCR, which can be identified independently [(6)].

We have developed real time PCR assays to detect tetracycline resistance genes in Gram-negative bacteria. The assay was developed as a multiplex SYBR Green I assay using the Roche Lightcycler and multi-melting peak analysis followed by a specific 5' nuclease assay. By performing simultaneous PCRs for *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G) and *tet*(H) in the same reaction tube with SYBR Green I, we were able to independently identify the different resistance determinants. The SYBR Green I assay then followed by a specific probe used to quantitatively confirm the previous results in a given specimen based on the TaqMan technology. This new real-time PCR allows the amplification and detection to take place in less than 1 hour and therefore significantly accelerates the screening of clinical specimens and its sensitivity.

METHODS AND RESULTS

Primers were designed for PCR amplification of seven different classes of *tet* genes commonly found in Gram-negative organisms based on the published sequence [(7)]. A combination of primer pairs were used in a multiplex PCR reaction with SYBR Green I to detect a group of *tet* genes (including A, B, C, D, E, G and H), (Figure 1). Based on the melting peak analysis of SYBR Green I PCR result, we could preliminarily determine the class of Tet resistance determinant that gave the positive signal. We designed specific TaqMan primers and probes using Primer Express software (ABI-Applied Biosystems , Foster City, CA) for further confirmation of each class of *tet* determinant (Figure 2). Positive results were determined by comparing the Ct (crossing point) in a class-specific 5' nuclease test. We screened forty-eight clinical isolates from different countries with those two tests. In 37 samples, the 5' nuclease assay confirmed the identity of the Tet resistant determinant shown in SYBR Green I multiplex PCR reaction (Figure 3, 4 and 5). 5 samples confirmed late belong to one uncommon *tet* gene (Tet J resistance determinant) in Gram-negative bacteria. Finally 6 samples were not identified using our current primer and probe sets (Table 1).

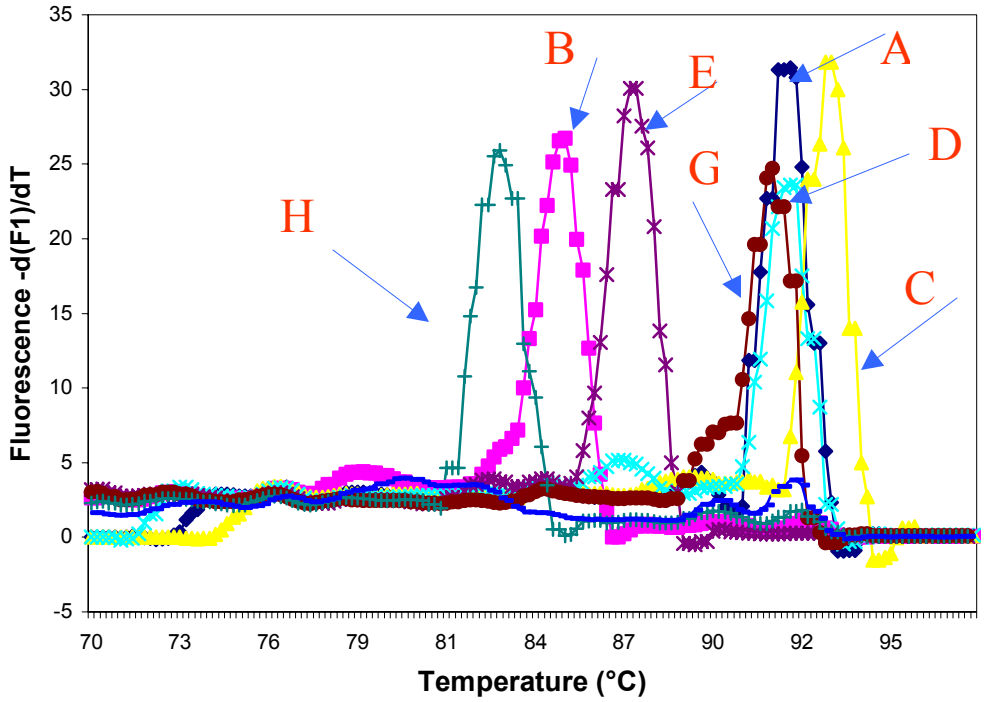

Figure 1. SYBR Green I detection of Tet resistance determinants using multi-melting peak analysis and LightCycler

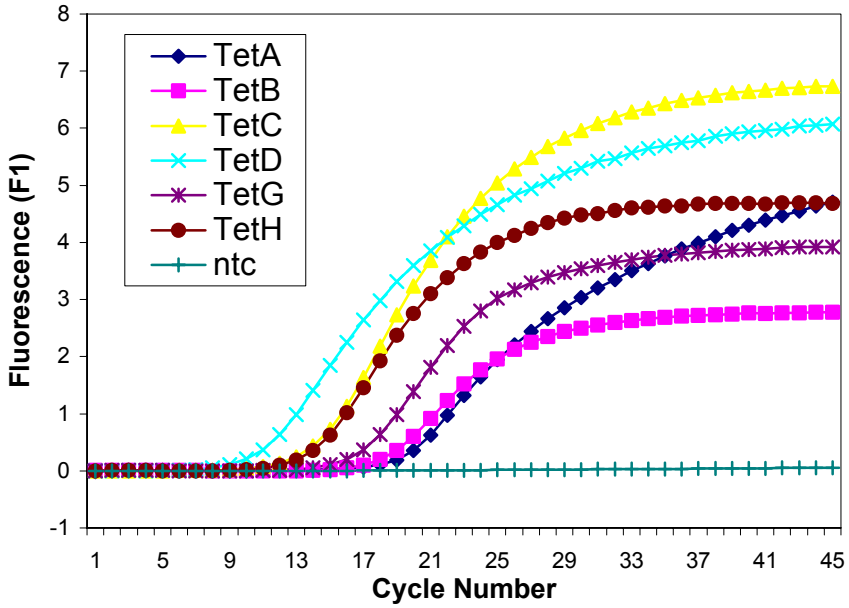

Figure 2. TaqMan assays for the specific identification of Tet resistance genes in Gram- negative bacteria.

In Figure 1,each single reaction had seven primer sets present (for class A, B, C, D, E, G and H) and only one template or water as a negative control (NTC). Thermocycling conditions were 45 cycles of 95°C 1 sec, 58°C 10 sec and 72°C for 20 sec. The melting peak analysis wasconducted from 65°C to 95°C at 0.1°C per sec. Thesamplesusedhere are 10 ng purified DNA.

In Figure 2, the probe used in each reaction is indicated in the sample label. Each sample contained homologous template. The reactions were ran for 45 cycles of denaturation at 94°C 0 sec followed by annealing/elongation at 58°C for 20 sec. The samples used here are 10ng of purified DNA. The probes did not show cross reactivity with non-homologous templates.

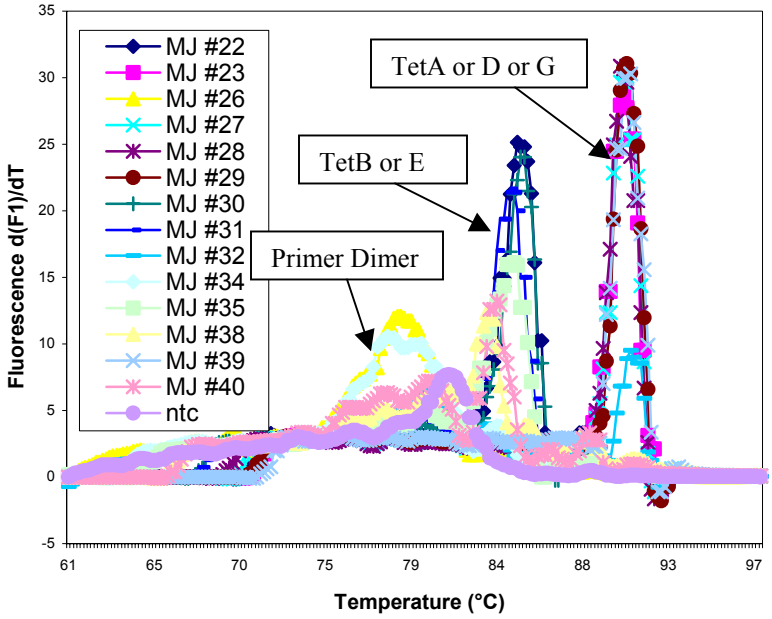

Figure 3. Screen of clinical samples for tetracycline resistance with the SYBR Green I multiplex PCR reaction.

In Figure 3, each single reaction had seven primer sets present (for class A, B, C, D, E, G and H) and only one template or water as a negative control (NTC). Samples used were 2.5ul lysate supernatant prepared by Prepman Ultra Sample Preparation Reagent (ABI-Applied Biosystems , Foster City, CA). Thermocycling conditions were 45 cycles of 95°C 1 sec, 58°C 10 sec and 72°C for 20 sec. The melting peak analysis was conducted from 65°C to 95°C at 0.1°C per sec.

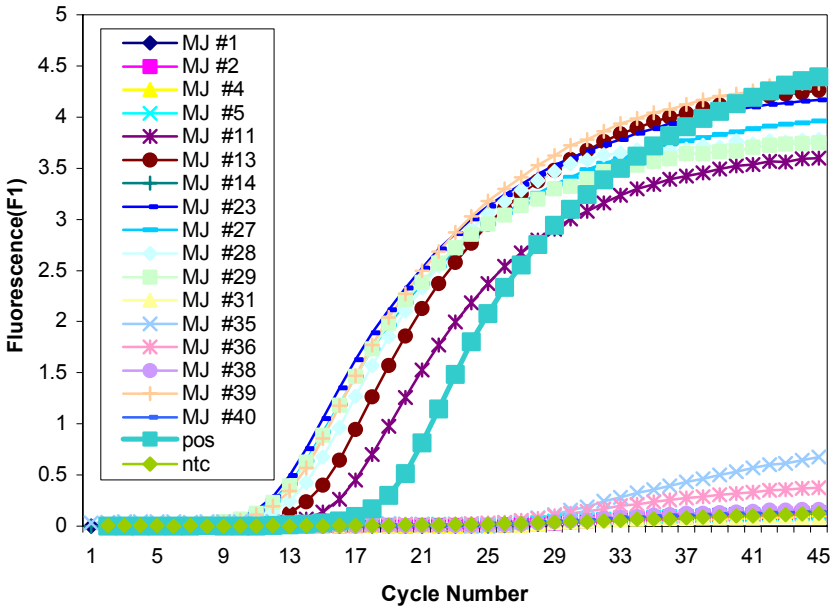

Figure 4. Confirmatory test of clinical isolates with TaqMan assay using class-specific primers and probe.

In Figure 4, in this example the Tet A specific probe was used. The reactions were run for 45 cycles of 94°C 0 sec followed by annealing/elongation at 58°C for 20 sec. Other probes reacted with samples only when homologous template was present. These tests confirmed the Tet class suggested in the multiplex assay (Figure 3).

Table 1. Summarization of clinical isolates test results with SYBR Green I screen and TaqMan confirmation*

CLASSIFICATION	SYBR REEN I SCREEN	TaqMan CONFIMATION
Tet A	13**	13
Tet B	24***	24
Tet D		1
	1	
Tet J	5	
Unkown	6	

* A total of 48 clinical isolates from Cobra Gold and University of Maryland were tested. The samples shown in the table were identified by both assays. Among these samples, one contains both A and B *tet* genes, one contains both B and J resistance determinants. Another 5 samples were later found to encoded to *tet* (J) gene. Finally, 6 Tet resistant isolates were not identified with our current primer and probe sets.

** One sample contains both A and B *tet* genes

*** One sample contains both A and B *tet* genes, one contains both B and J *tet* genes.

In Figure 5, in this example the Tet B specific probe was used. The reactions were run for 45 cycles of 94°C 0 sec followed by annealing/elongation at 58°C for 20 sec. Other probes reacted with samples only when homologous template was present. These tests confirmed the Tet class suggested in the multiplex assay (Figure 3).

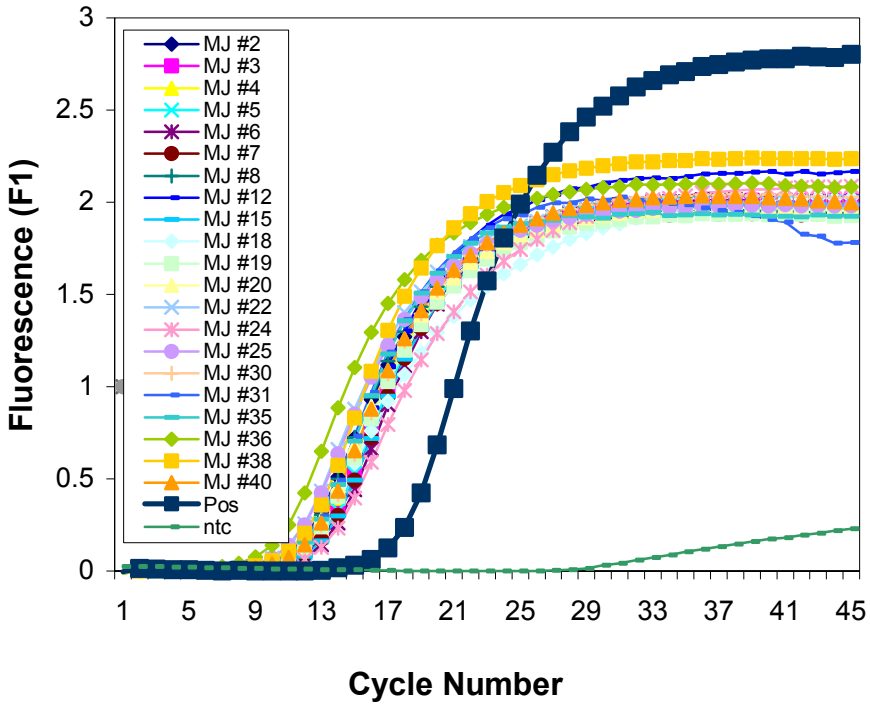

Figure 5. Confirmatory test of clinical isolates with TaqMan assay using class-specific primers and probe.

CONCLUSIONS

In conclusion, we describe a two-step real-time capillary PCR method to detect seven different classes of *tet* gene in Gram-negative bacteria. The use of a quick and reliable multiplex PCR with SYBR Green I melting point analysis could assist in the preliminary identification of tetracycline resistance gene resulting in a saving of both time and labor in screening large number of strains. The follow up with class-specific TaqMan reaction made a quick and final diagnosis. This approach made it possible to rapidly and reliably detect tetracycline resistance genes. And it also could be easily extended to other classes of antibiotic resistance gene detection.

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